OXFORD UNIVERSITY PRESS | Human Reproduction Update

The estrogen-macrophage interplay in the homeostasis of the female reproductive tract

Journal:	*Human Reproduction Update*
Manuscript ID	HRU-17-0057.R2
Manuscript Type:	Review
Date Submitted by the Author:	n/a
Complete List of Authors:	Pepe, Giovanna; Universita degli Studi di Milano, Center or Excellence on Neurodegenerative Diseases, Departiment of Pharmacological and Biomolecualr Sciences Locati, Massimo; Humanitas Clinical Research Center and Università degli Studi di Milano, Department of Medical Biotechnologies and Translational Medicine Della Torre, Sara; Universita degli Studi di Milano, Center or Excellence on Neurodegenerative Diseases, Departiment of Pharmacological and Biomolecualr Sciences Mornata, Federica; Universita degli Studi di Milano, Center or Excellence on Neurodegenerative Diseases, Departiment of Pharmacological and Biomolecualr Sciences Cignarella, Andrea; Università degli Studi di Padova, Department of Pharmaceutical Sciences Maggi, Adriana; Universita degli Studi di Milano, Center or Excellence on Neurodegenerative Diseases, Departiment of Pharmacological and Biomolecualr Sciences Vegeto, Elisabetta; Universita degli Studi di Milano, Center or Excellence on Neurodegenerative Diseases, Departiment of Pharmacological and Biomolecualr Sciences
Keywords:	ESTROGEN, macrophages, female reproductive tract, inflammation, ovarian cancer, ENDOMETRIOSIS

TITLE

The estrogens-macrophage interplay in the homeostasis of the female reproductive tract

RUNNING TITLE

Macrophages regulate female reproductive tissues by adapting to estrogens signal

AUTHORS

Giovanna Pepe[1]

Massimo Locati[2,3]

Sara Della Torre[1]

Federica Mornata[1]

Andrea Cignarella[4]

Adriana Maggi[1]

Elisabetta Vegeto[1*]

AFFILIATIONS

[1]Center of Excellence on Neurodegenerative Diseases, Department of Pharmacological and Biomolecular Sciences, University of Milan, via Balzaretti, 9 20133, Milan, Italy

[2]Humanitas Clinical and Research Center and [3]Department of Medical Biotechnologies and Translational Medicine, University of Milan, via fratelli Cervi, 20089, Segrate, Italy

[4]Department of Medicine, University of Padua, Largo Meneghetti 2 35131, Padua, Italy

*Corresponding author (elisabetta.vegeto@unimi.it)

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ABSTRACT

Background. Estrogens are known to orchestrate reproductive events and to potentiate the immune system against infections and tissue damage. Recent findings suggest that, in the absence of any danger signal, estrogens trigger the physiological expansion and functional specialization of macrophages, which are immune cells that populate the female reproductive tract (FRT) and are increasingly being recognized to participate in tissue homeostasis beyond their immune activity against infections. Although estrogens are the only female gonadal hormones that directly target macrophages, a comprehensive view of this endocrine-immune communication and its involvement in the FRT is still missing.

Objective and rationale. Recent accomplishments encourage a revision of the literature on the ability of macrophages to respond to estrogens and induce tissue-specific functions required for reproductive events, with the aim to envision macrophages as key players in FRT homeostasis and mediators of the regenerative and trophic actions of estrogens.

Search methods. We conducted a systematic search using PubMed and Ovid for human, animal (rodents) and cellular studies published until 2018 on estrogen action in macrophages and the activity of these cells in the FRT.

Outcomes. Our search allowed the appreciation of the remarkable ability of macrophages to activate biochemical processes in response to estrogens in cell culture experiments. The distribution at specific locations, interaction with selected cells and acquisition of distinct phenotypes of macrophages in the FRT, as well as the cyclic renewal of these properties at each ovarian cycle, demonstrate the involvement of these cells in the homeostasis of reproductive events. Moreover, current evidence suggests the association between the estrogen-macrophage signaling and the generation of a tolerant and regenerative environment in the FRT, although a causative link is still missing.

Wider applications. Dysregulation of the functions and estrogen responsiveness of FRT macrophages may be involved in infertility and estrogens and macrophage-dependent gynecological diseases, such as ovarian cancer and endometriosis. Thus, more research is needed on the physiology and pharmacological control of this endocrine-immune interplay.

KEYWORDS

Estrogens; macrophages; female reproductive tract; inflammation; ovarian cancer; endometriosis

Introduction

The fluctuations in estrogen levels that occur during the menstrual cycle in women regulate innate defensive mechanisms against pathogen invasion and modify the susceptibility to inflammatory diseases, such as atherosclerosis, ischemia or autoimmune pathologies; these immune mechanisms have been proposed to explain, at least in part, the different immune responses in females as compared to males (Jørgensen, 2015). Such immunomodulatory activity has been ascribed, at least in part, to the direct activity of estrogens in macrophages, while other sex steroid hormones, androgen and progesterone, show either little or null effect (Kovats, 2015).

Macrophages are important players in innate immunity and their deranged activation has effects in human inflammatory pathologies. Beyond immunity, recent investigations demonstrated novel functions for macrophages, which are dictated by a vast array of physiological cues and in response to specific regulatory interactions that macrophages establish with the specific cell types and matrix components within tissues (Gordon and Plüddemann, 2017). Indeed, macrophages were proved to act in diverse organs of the female reproductive tract (FRT) by non-immune processes and recently shown to undergo a specific phenotypic adaptation in response to estrogens and estrogens-regulated mediators that promotes immune tolerance and tissue remodeling (Pollard *et al.*, 1998; Pepe, Braga, *et al.*, 2017). These novel data encourage a revision of the molecular and biological details of the macrophage response to estrogens and the evidence on the distribution and activity of these cells in the FRT, with insight into the relevance of this endocrine-immune interplay in FRT homeostasis and diseases.

Macrophage biology

Origins and renewal

Macrophages in adult tissues may have dual origin. During fetal life, embryonic progenitors migrate into developing organs to constitute the resident population of macrophages that can self-replenish throughout life. Tissue macrophages also derive from hematopoiesis, as blood monocytes may infiltrate into tissues and differentiate into mature cells (Schulz *et al.*, 2012; Sieweke and Allen, 2013; Yona *et al.*, 2013). Self-

renewal of tissue resident macrophages is regulated by the lineage specific growth factor, macrophage-colony stimulating factor (CSF1), as well as by immune and endocrine signals, such as interleukin-4 (IL-4), IL-33 and estrogens, in a tissue-specific manner (Hashimoto *et al.*, 2013; Jackson-Jones *et al.*, 2016; Jenkins *et al.*, 2013; Pepe, Braga, *et al.*, 2017; Pepe, De Maglie, *et al.*, 2017; Tagliani *et al.*, 2011). Multiple physiological signals, including CSF1 and the chemokines Monocyte Chemoattractant Protein-1 (MCP-1/CCL2) and Macrophage Inhibitory Protein 1-α (MIP-1α/CCL3), are clearly involved in the recruitment of monocytes (Pollard *et al.*, 1987, 1998; Robertson *et al.*, 1996; Wood *et al.*, 1997; Long *et al.*, 1998; Klotz *et al.*, 2002; Moldenhauer *et al.*, 2010; Wheeler *et al.*, 2018). The population of macrophages in the FRT is maintained by both the self-renewal and monocyte recruitment, as also reported for other organs such as spleen and kidney. Expansion and recruitment of FRT macrophages occur under the influence of chemoattractive and proliferative signals that are released by FRT cells in response to endocrine and physiological stimuli, including estrogens. Thus, beyond their direct activity estrogens indirectly regulate macrophage number by increasing the expression of cytokines and chemokines in epithelial cells of the uterus and oviducts. Indeed, ablation of the genes coding for these mediators triggers defective macrophage and reproductive functions in animal models (Lavin *et al.*, 2014; Pollard *et al.*, 1987; Schulz *et al.*, 2012).

Physiologic functions of macrophages

We here summarize the main physiological activities that are routinely carried out by macrophages located in various tissues, while more specialized functions related to estrogen signaling and the FRT are discussed in Sections 2 and 3.

Inflammation, immune activation and tissue homeostasis

In response to bacterial or viral infections macrophages acquire a classical activation phenotype, named M1 in analogy with T-helper nomenclature, characterized by the production of inflammatory mediators such as cytokines, reactive oxygen species and arachidonic acid metabolites, which sustain inflammation and kill invading microbes. Instead, stimuli such as IL-4 and IL-13, together with tissue resident signals, lead

macrophages to acquire an "alternative" or M2 activation state, which is involved in tissue remodeling (Wynn and Vannella, 2016; Minutti *et al.*, 2017). Though M1-M2 polarization has been shown to occur *in vivo*, this classification should only be considered a schematic representation of a spectrum of intermediary phenotypes induced by the combinatorial effects of stimuli and other cell types present in the microenvironment (Xue *et al.*, 2014).

Macrophage phenotypic adaptations are mediated by specific transcription factors, such as Nuclear Factor-Kappa enhancer of activated B cells (NF-κB) that is crucial for the expression of genes linked to the M1 inflammatory response, and CCAAT-enhancer-binding protein-b (C/EBPb), Kruppel-like Factor-4 (KLF4) and the transcriptional repressor KLF11 involved in M2 gene expression (Bouhlel *et al.*, 2007; Takeda *et al.*, 2010; Lawrence and Natoli, 2011; Liao *et al.*, 2011; Pello *et al.*, 2012). Interestingly, some of these transcription factors are also highly expressed in the FRT and involved in reproductive tissue pathologies (Navarro *et al.*, 2012; Daftary *et al.*, 2013). Distinct phenotypes also correspond to specific adaptations of macrophage energy metabolism, so that resting and M2 macrophages produce energy by the potentiation of oxidative phosphorylation and tricarboxylic acid cycle, while M1 activation is associated with higher rates of glycolysis (Vats *et al.*, 2006; Palsson-McDermott and O'Neill, 2013).

The phenotypic adaptation of macrophages is crucial for communicating to the surrounding cells and the extracellular matrix (ECM; Wynn and Vannella, 2016). Classically activated macrophages sustain matrix destruction through the secretion of proteases, such as matrix-metalloproteinases (MMPs) and cathepsin K, and the increased expression of receptors for matrix proteins, such as Mac1 for fibrinogen (Adhyatmika *et al.*, 2015). On the other hand, alternatively activated cells produce anti-inflammatory and pro-fibrotic mediators, such as Transforming Growth Factor-β1 (TGF-β1), C Chemokine Ligand 18 (CCL18) and Resistin-Like Molecule α (RELM-α), which promote proliferation of surrounding cells and matrix synthesis and deposition (Knipper *et al.*, 2015; Liu *et al.*, 2004). Chronically activated inflammatory macrophages may lead to tissue degeneration, while the uncontrolled activation of the M2 phenotype is a pro-fibrotic process that drives tissue fibrosis and non-healing wounds (Minutti *et al.*, 2017; Wynn and Vannella, 2016). The function of macrophages in the FRT is clearly and demonstrably controlled by macrophage-specific

regulators that are locally synthesized by cells, such as uterine epithelia, also under the influence of estrogens (Moldenhauer *et al.*, 2010).

Phagocytosis

Macrophages recognize, engulf and degrade microorganisms or "self" cells, or parts of them, through the engagement of specific phagocytic receptors. The phagocytosis of a pathogen is activated by the ability of pattern-recognition receptors (PRRs) to bind to specific molecules of the pathogen cell wall, such as mannans in yeasts and lipopolysaccharide (LPS) in bacteria (Weiss and Schaible, 2015). On the other hand, phagocytosis of self-cells is a natural homeostatic process in cell turnover induced by "eat-me" signals, such as phospholipid phosphatidylserine, and inhibited by "don't-eat-me" signals, such as sialic acid, which are recognized by specific scavenger receptors abundantly expressed by macrophages (Arandjelovic and Ravichandran, 2015; Gordon and Plüddemann, 2018). Importantly, PRR activation is coupled with the production of pro-inflammatory molecules, while engulfment of apoptotic cells transmits an immunosuppressive signal in macrophages to curtail inflammation and promote tissue remodeling.

Estrogen signaling and macrophage responses

Biosynthesis. Gonadal steroidogenesis is mediated by a cooperative interaction between thecal and granulosa cells, known as the "two-cell" model, which is tightly regulated in time and space by neuroendocrine signals (Hillier *et al.*, 1994). Under the influence of luteinizing hormone (LH), steroidogenesis begins in thecal cells, which take up large amounts of cholesterol via the low density lipoprotein receptor (LDLR) and convert it into shorter intermediates. These lipophilic molecules diffuse through the basal lamina and infiltrate granulosa cells, which instead receive no blood supply and have minimal levels of LDLR and cholesterol-modifying enzymes, except for the aromatase enzyme, the last enzyme in estrogens biosynthesis that is expressed under the control of follicle stimulating hormone (FSH). This neuroendocrine system generates the typical temporal profile of blood estrogen levels, which gradually increase during the early and mid-proliferative phases until sharply peaking and immediately declining at the end of the proliferative phase before ovulation, which is triggered by the LH surge at mid-

cycle; estrogens synthesis is then sustained by luteinizing cells of the *corpus luteum* in the secretory phase and decreases during luteolysis. The most abundant and active estrogen is 17β-estradiol (E_2). Macrophages are physically confined to the thecal cell layer in the growing follicle, while they gain contact with luteinizing cells after ovulation, suggesting a specific role in cholesterol handling and steroidogenesis, as further described in section 3.

The molecular mechanism of estrogen action

Estrogen receptors. Estrogen action is mediated by two intracellular estrogen receptors (ERs), namely ESR1 (ERα) and ESR2 (ERβ), and by the G protein-coupled estrogen receptor 1 (GPER1), a plasma membrane protein which binds E_2 and ER agonists/antagonists with a reduced affinity (10-100 fold and 1,000-fold lower, respectively) than that of intracellular ERs (Petrie *et al.*, 2013; Thomas *et al.*, 2005). Human and mouse macrophages express the *Esr1* and *Gper1* genes, while expression of ERβ and progesterone receptor (PR) in macrophages is controversial (Lambert *et al.*, 2004; Rettew *et al.*, 2010; Ribas *et al.*, 2011a; Vegeto *et al.*, 2004; Villa *et al.*, 2016). To clarify this issue, we searched in public repository sites for transcriptomics datasets obtained by RNA sequencing of mouse and human resting macrophages and report the data relative to steroid receptors in Table 1. ERβ and PR are not detectable, the androgen receptor (AR) is expressed at low levels, while ERα and GPER1 mRNAs are present at different absolute values among datasets, probably due to the sensitivity of the methodology used. However, their relative abundance remains unchanged when considered in relation with the house-keeping gene, ribosomal protein lateral stalk subunit P0 (*Rplp0*), or the *Nr3C1* gene coding for the glucocorticoid receptor (GR), whose expression and activity are widely described in macrophages (Martinez *et al.*, 2006; Pepe, Braga, *et al.*, 2017). Thus, in line with the general consensus, this analysis supports the conclusion that estrogen action in macrophages is mainly mediated by ERα and GPER1 under physiological conditions, and that these cells are not able to respond to progesterone, at least through a receptor-mediated mechanism under physiological conditions.

Estrogen receptor expression may be regulated by genetic or epigenetic mechanisms induced by estrogen itself or by pathological conditions such as inflammation, obesity and high fat diet in the case of macrophage ERα (Ribas *et al.*, 2011; Villa *et al.*, 2015) or endometriosis for uterine GPER1 and ERβ (Adams

et al., 2007; Han *et al.*, 2015; Heublein *et al.*, 2013; Nasu *et al.*, 2011; Renthal *et al.*, 2013; Ribas *et al.*, 2011b; Villa *et al.*, 2015). Despite being the most abundant sex steroid receptor in macrophages, ERα levels are lower than in breast epithelial cells, possibly due to a cell-specific usage of diverse promoter regions within the *Esr1* gene (Murphy *et al.*, 2009). Thus, the unique expression of ERα among sex steroid receptors of in macrophages and its liability to regulation suggest a physiologic role for this receptor in the endocrine regulation of macrophage responses.

Regualtion of receptor activity. As summarized in Figure 1, ERα is a transcription factor that is activated by estrogens to regulate target gene transcription by directly binding to target gene promoters and recruiting transcriptional co-regulators, or to interfere with the activity of other transcription factors. Estrogen-activated ERα and GPER1 also regulate cytoplasmic effectors that modulate intracellular lipids, Ca^{2+} or cAMP levels (Smith and O'Malley, 2004; Revankar *et al.*, 2005; Deroo and Korach, 2006; Levin, 2015). While target genes expression changes within hours, non-genomic responses occur within minutes since the estrogen surge. The response to estrogens varies in different tissues as a result of cell-specific differences in the expression levels and activity of hormone receptors and their co-regulators. Hormonal responses need also to be considered in a dynamic view, since estrogen levels progressively increase during the proliferative phase of the ovarian cycle and induce later responses triggered, as in a cascade model, by the initial estrogen-responsive targets (Della Torre *et al.*, 2011). In macrophages, estrogens were shown to regulate gene expression through ERα and to induce non-genomic responses mediated by both ERα and GPER1 (Cote *et al.*, 2015; Frazier-Jessen and Kovacs, 1995; Ghisletti *et al.*, 2005; Guo *et al.*, 2002; Hsieh *et al.*, 2009; Liu *et al.*, 2013; Murphy *et al.*, 2010; Pepe, Braga, *et al.*, 2017; Qian *et al.*, 2015; Rettew *et al.*, 2010; Suzuki *et al.*, 2008). The dose and time-dependent mechanisms of action are particularly relevant for peritoneal organs, where estrogen levels are higher than in peripheral tissues (Loumaye *et al.*, 1985; Manolopoulos *et al.*, 2001).

Estrogen receptor activity can be switched on or off by other endogenous molecules. Receptor activation may be triggered by intracellular kinases that are activated by diverse signals, including inflammatory cytokines, and induce modifications in the ERα conformation resulting in receptor-mediated genomic

responses (Stellato *et al.*, 2016)(Stender *et al.*, 2017). Moreover, progesterone is known to oppose estrogen actions in the uterus and vagina through the differentiation from proliferative to secretory endometrial cells, production of less potent estrogens and formation of vaginal mucus that hinders sperm survival (Patel *et al.*, 2015). The opposed activity is less defined in *corpus luteum*, as both progesterone and estrogen participate in luteal functions and regression, while it does not seem to occur in macrophages, as these cells do not express PRs (see Table 1).

Constitutive and macrophage-specific ablation of ER. ER knock-out models showed that ERα is responsible for the effects of estrogens in FRT physiology, with ERβ being important in ovulation and GPER1 dispensable for fertility and reproduction (Dupont *et al.*, 2000; Hamilton *et al.*, 2014; Hewitt *et al.*, 2016). Transgenic mice also confirmed the primary role of ERα in macrophage responses to estrogens in various tissues, including brain, skin, lung and peritoneum, although GPER1 may also be involved (Vegeto *et al.*, 2003, 2010; Garidou *et al.*, 2004; Lambert *et al.*, 2004; Campbell *et al.*, 2014; Wei *et al.*, 2016; Pepe *et al.*, 2017). Animal models carrying myeloid-specific ablation of ERα unraveled its contribution in maintaining key macrophage functions, such as oxidative metabolism, phagocytosis, cholesterol uptake and phenotypic activation (Calippe *et al.*, 2010; Campbell *et al.*, 2014; Ribas *et al.*, 2011). However, indications on the reproductive phenotype are only available for the myeloid-specific ERα deficiency (MACER) mice, which were reported to be fertile but also to develop liver, metabolic and adipose abnormalities reminiscent of dysmetabolic traits observed in women with polycystic ovarian syndrome (PCOS), who also develop subfertility and menstrual irregularities (Ribas *et al.*, 2011a; Teede *et al.*, 2010). Interestingly, when exposed to insults such as caloric restriction, metabolic imbalance or infections, different transgenic female mice displayed a subfertility phenotype, described by anestrous, lengthened ovarian cycles or reduced number of post-implantation embryos, while maintaining a fertile phenotype under unstimulated conditions (Martinez de la Torre *et al.*, 2007; Della Torre *et al.*, 2016). Thus, subtle alterations in reproductive processes should be addressed to define the relevance of estrogen action in macrophages and precursor cells within the FRT, also considering that compensatory mechanisms may substitute for the deletion of a transcription factor involved in phenotype specialization, such as ERα.

Macrophage responses to estrogen

Our understanding of the functional interplay between estrogens and macrophages grew in parallel with the acquisition of knowledge on novel aspects of macrophage biology, such as ontogenesis, self-renewal, function specialization and lineage heterogeneity. Thus, from initial observations using classic inflammatory paradigms showing the anti-inflammatory activity of estrogen, subsequent analysis demonstrated a hormone effect also on macrophage reparative phenotype, while only recently estrogen was envisioned as a physiologic signal that may regulate macrophage reactivity *per se* (Bruce-Keller *et al.*, 2000; Campbell *et al.*, 2014; Salem, 2004; Vegeto *et al.*, 2001; Villa *et al.*, 2015). In the hypothesis of conceiving macrophages as key messengers in FRT homeostasis orchestrated by estrogens, the following paragraphs discuss macrophage responses to estrogens beyond immunity against infections, as summarized in Figure 1.

***Proliferation*.** E_2 has been involved in macrophage proliferation *via* either direct mechanisms or increased production of growth factors, such as EGF and IGF1, by non-macrophage cells (Pollard *et al.*, 1987; Klotz *et al.*, 2002; Pepe *et al.*, 2017). It still needs to be verified whether the renewal of resident macrophages cyclically occurring in the FRT during the ovarian cycle, particularly in the proliferative phase, also involves a direct proliferative effect of estrogens.

Immune polarization and extracellular communication. A comprehensive description of the genomic responses induced by the estrogen surge in peritoneal macrophages of female mice showed the dynamic and variegated adaptation of macrophages to the hormonal signal *per se*, in the absence of pathological or inflammatory stimuli, which occurs through the regulation of early and late genes, such as *Vegf* and *IL10* (Pepe *et al.*, 2017). Under inflammatory conditions, estrogens have been proposed to anticipate both the onset and termination and to enhance the potency of the inflammatory response driven by macrophages and to favor the transition towards an M2-like phenotype, in line with improved outcome of inflammatory responses in female mice and humans (Bolego *et al.*, 2013; Rathod *et al.*, 2017; Scotland *et al.*, 2011; Toniolo *et al.*, 2015; Villa *et al.*, 2015). These effects have been reconciled with genomic and cytoplasmic mechanisms induced by estrogen-activated ERα and GPER1. The activity of M1 or M2 stimuli on the

expression of genes, such as *MMP9*, Tumor Necrosis Factor-α (*TNF-α*), *IL-1β* and *MIP2* or arginase 1 (*ARG1*), Transglutaminase 2 (*TGM2*) and *RELMα*, respectively, is modified by the presence of estrogens according to the tissue of origin of macrophages or the cell line used (Campbell *et al.*, 2014; Cote *et al.*, 2015; Frazier-Jessen and Kovacs, 1995; Ghisletti *et al.*, 2005; Pervin *et al.*, 1998; Ribas *et al.*, 2011a; Ruh *et al.*, 1998; Vegeto *et al.*, 2004). E_2-activated ERα may also interfere with the activity of transcription factors that drive macrophage polarization, while the effects on energy consumption widely described for other target cells are still unknown in macrophages (Dai *et al.*, 2009; Duckles *et al.*, 2006; Ghisletti *et al.*, 2005; Mattingly *et al.*, 2008; Villa *et al.*, 2015; Wang *et al.*, 2001; Xing *et al.*, 2012).

Studies focused on ECM remodeling, in particular on the wound healing process, showed that estrogens fasten tissue repair by contributing to epithelial, collagen and vascular remodeling through a direct activity on macrophages and the increased secretion of: i) tissue repair molecules, such as RELM-α (Ashcroft *et al.*, 1997; Campbell *et al.*, 2014; Liu *et al.*, 2004); ii) proteases, such as matrix metalloproteinases (MMPs) and cathepsins, and their inhibitors (Rochefort *et al.*, 2001; Vegeto *et al.*, 2001); iii) the TGM2 enzyme, a conserved M2 marker highly expressed by human and murine macrophages in Th2-driven pathologies, involved in matrix protein crosslinking, clearance of apoptotic cells and promotion of an anti-inflammatory phenotype (Eligini *et al.*, 2016; Martinez *et al.*, 2013; Pepe, Braga, *et al.*, 2017; Ribas *et al.*, 2011a); iv) Fibroblast Growth Factor (FGF) and VEGF, through the involvement of both ERα and GPER1 (McLaren *et al.*, 1996; Kanda and Watanabe, 2002; Khan *et al.*, 2005; Pepe *et al.*, 2017). Thus, matrix and microenvironment remodeling by macrophages appears to be potentiated by estrogen, as initially demonstrated in an animal model of peritoneal adhesion formation in which estrogen administration reduced connective tissue deposition (Frazier-Jessen *et al.*, 1996).

***Phagocytosis*.** In relation with the nature of the activating signal, estrogens are able to modulate the phagocytic activity of macrophages. As shown for immune polarization, estrogens exert opposite effects in the presence of M1 or M2 stimuli, reducing the effects of LPS or β-amyloid on phagocytosis and expression of receptors, such as CD14 and scavenger receptor-A (SR-A), or enhancing the phagocytosis of parasite or immunoglobulin-coated cells, possibly *via* increased expression of macrophage receptors for “eat-me-

signals" (Bruce-Keller *et al.*, 2000; Hsieh *et al.*, 2009; Ning *et al.*, 2016; Saia *et al.*, 2015; Vegeto *et al.*, 2004, 2006; Yu *et al.*, 2014; Zhang *et al.*, 2015).

Iron homeostasis. Iron is an essential cofactor for several metabolic processes within cells, yet it is extremely toxic if not handled properly by tissues. Resident macrophages process large amounts of iron through the expression of receptors that import protein-bound iron, such as the transferrin receptor-1 (TFRC) and CD163, or free extracellular iron, such as Six-Transmembrane Epithelial Antigen of Prostate 3 (STEAP3) and Divalent Metal Transporter-1 (DMT1/Slc11a2) (Kohyama *et al.*, 2009; Haldar *et al.*, 2014; Korolnek and Hamza, 2015). Inside macrophages, iron may be used for the cell metabolic demand, stored as ferritin-bound form or exported by ferroportin-1 (FPN). Iron efflux is negatively regulated by hepcidin, an hepatic hormone that induces FPN endocytosis and degradation (Nemeth *et al.*, 2004). M1 macrophages develop an iron-sequestering phenotype that restricts extracellular iron availability for pathogens, while an iron-releasing phenotype that sustains the growth of surrounding cells is ascribed to alternative activation of macrophages through the expression of genes involved in iron turnover, mobilization and release (Cairo *et al.*, 2011). Estrogens increase cellular iron uptake *via* the positive regulation of TFRC, iron binding proteins and transporters as well as by a negative effect on hepcidin expression in liver (Yang *et al.*, 2012). In the FRT, estrogens induce the temporally coordinated expression of genes related with iron homeostasis, such as the iron delivery and exporter proteins, lactotransferrin (LTF), lipocalin-2 (LCN2) and FPN, respectively. By contrast, hormone action in macrophages has been poorly investigated, with some contrasting results depending on the specific macrophage population analyzed (Campesi *et al.*, 2012; Hamad and Awadallah, 2013; Pentecost and Teng, 1987; Huang *et al.*, 1999; Pepe, Braga, *et al.*, 2017; Qian *et al.*, 2015; Stuckey *et al.*, 2006; Yang *et al.*, 2012).

Hemostasis and beyond. Macrophages are a source of factors for coagulation and complement activation that contribute to thrombin and fibrin formation and platelet aggregation (Boyce *et al.*, 2015; van der Meer *et al.*, 2014). In turn, molecules of the hemostatic system directly bind to macrophages through specific receptors and induce responses such as inflammation, angiogenesis, phagocytosis and matrix remodeling. For instance, thrombin and fibrin remain trapped in the perivascular space after vessel rupture and from

this site they bind to tissue resident macrophages and induce the production of inflammatory and fibrinolytic mediators that are required for tissue healing (Gratchev *et al.*, 2001; Davalos *et al.*, 2012). Although oral estrogen therapy is known to induce a pro-coagulant state through the transcriptional regulation of hemostasis genes in liver, additional details on how estrogens act on FRT hemostasis are still lacking.

Cholesterol metabolism. Cholesterol is transported in blood in the form of cholesterol esters (CEs) mainly bound to LDL and its cellular intake occurs through endocytosis mediated by LDL-R. Within endosomes/lysosomes, CEs are hydrolyzed to release free cholesterol, which may be used for membranes synthesis, stored in cytoplasmic lipid droplets continuously processed by hydrolysis and re-esterification, or excreted by efflux systems (Brown and Goldstein, 1983). Incorrect cholesterol handling may transform macrophages into foam cells that sustain atherosclerotic lesions formation (von Eckardstein, 1996). Consistent evidence showed that E_2 reduces the uptake and favors the efflux of cholesterol by macrophages under inflammatory conditions, also by down-regulating the expression of scavenger receptors CD36 and SR-A (Allred *et al.*, 2006; Corcoran *et al.*, 2011; McCrohon *et al.*, 1999; Napolitano *et al.*, 2001; Rayner *et al.*, 2008; Shchelkunova *et al.*, 2013; Tomita *et al.*, 1996; Vegeto *et al.*, 2006; Wilson *et al.*, 2008). Human and mouse macrophages were shown to express steroidogenic enzymes *in vitro*, depending on the tissue of origin (Rubinow, 2018).

Circadian rhythm. Circadian rhythmicity is driven by a molecular clock composed of a transcriptional regulator complex that is mainly activated by daily brain signals. However, an intrinsic molecular clock in peripheral tissues also works independently of brain inputs and its disruption is associated with chronic pathologies. In particular, clock gene expression in the ovaries is involved in the timing of reproductive events and in fertility, as further discussed in section 3 (Mereness *et al.*, 2016; McAlpine and Swirski, 2016; Sen and Sellix, 2016). Macrophages express circadian clock genes also independently from the brain pacemaker (Boivin *et al.*, 2003; Keller *et al.*, 2009); interestingly, macrophage inflammatory responses follow circadian rhythmicity and require clock genes to efficiently take place (Spengler *et al.*, 2012; Oliva-Ramírez *et al.*, 2014; Nakazato *et al.*, 2017). Endogenous or pharmacological fluctuations of estrogens in

rodents have been shown to regulate the expression of clock genes, such as Periodic Circadian clock 1 (*Per1*) and *Per2*, in macrophages and in the FRT (Nakamura *et al.*, 2005, 2010; Zhu *et al.*, 2015; Wiggins and Legge, 2016; Pepe *et al.*, 2017).

The role of macrophages in the homeostasis of the female reproductive tract

The FRT is a peculiar site where the immune system is constantly balanced between aggression and tolerance towards the seminal fluid, fertilized egg and microorganisms as well as self-components and tissue remodeling. Indeed, macrophages in the FRT not only protect against infection but also participate in reproductive events through the physical and functional interaction with surrounding cells, matrix and fluids, similarly to macrophages that reside in brain, liver or lung (Gertig and Hanisch, 2014; Lavin *et al.*, 2014; Minutti *et al.*, 2017).

The number and function of FRT macrophages change in a precise temporal and spatial manner during the ovarian cycle. Target cells for estrogens include leukocytes of the FRT, which operate in synchrony with other cells to adapt to the oocyte fate (Givan *et al.*, 1997; Evans and Salamonsen, 2012). The paragraphs below summarize the evidence on macrophage distribution and functions in the ovaries, ovarian tubes, uterus and lower genital tract, as summarized in Figure 2, and the relevance of macrophages in ovarian and endometrial pathologies.

Macrophage-depleted animal models. An undisputed advance in the understanding of macrophage physiology is provided by mouse models that allow for the constitutive or conditional ablation of macrophages *in vivo*. Table 2 summarizes the reproductive and FRT phenotypes together with their drawbacks such as incomplete macrophage depletion, as in the case of clodronate or monoclonal antibodies targeting CSF1R (Van der Hoek *et al.*, 2000; MacDonald *et al.*, 2010; Sauter *et al.*, 2014), or developmental defects of the hypothalamus, occurring in mice bearing a null mutation in *Csf1* (*Csf1*^{op}/*Csf1*^{op}) or *Csf1r* gene knock-out, which alter reproductive functions independently of macrophage number in the adult FRT (Cohen *et al.*, 1999, 2002; Dai *et al.*, 2002). *CD11b-Dtr* transgenic mice, in which the diphtheria toxin receptor (DTR) is specifically expressed by CD11b-positive cells, may remove such

obstacles and allow for the acute and reversible reduction of macrophages in the entire organism including the FRT (Duffield *et al.*, 2005).

Macrophages in the ovaries

Cell distribution

Macrophages are preferentially located within the endocrine compartment of the ovary, where they change in number and function during the ovarian cycle, as summarized in Figure 2. While absent from the ovarian stroma and ovarian surface epithelium (OSE), macrophages appear in the theca cell layer and interstitial space of primary follicles at early stages of development (Wu *et al.*, 2004; Gaytán *et al.*, 2007). Macrophage cells number then gradually increases and sharply augments in thecal layers in preovulatory follicles (Brännström and Enskog, 2002; Van der Hoek *et al.*, 2000). Instead, macrophages are excluded from the granulosa cell compartment of antral follicles, while they are abundant in *corpora lutea*, reaching a peak at luteal regression, and in atretic follicles, where they are in contact with apoptotic granulosa cells (Wu *et al.*, 2004). Ovarian macrophages seemingly derive from monocytes supplied by blood that flows in the theca, and not granulosa, compartment of antral follicles and in the vastly vascularized *corpora lutea*; recruiting factors, such as CSF1, MCP-1/CCL2 and IL-33, are produced by ovarian and granulosa cells particularly in response to LH at ovulation (Hume *et al.*, 1984; Carlock *et al.*, 2014).

The preferential location of macrophages at specific microanatomical regions within the ovaries recalls that seen in endocrine organs, the pancreas and testis, for which more details are available on the role of macrophages in tissue homeostasis. In these organs, macrophages were shown to establish a symbiotic connection with endocrine and vascular cells, forming a functional unit that is essential for the correct production of insulin and androgens (Bhushan and Meinhardt, 2017; Calderon *et al.*, 2015; Cohen *et al.*, 1999; Turner *et al.*, 2011; Unanue, 2016). Whether macrophages are similarly relevant for the endocrine activity of the ovaries still needs to be defined. Conversely, it is also of interest that macrophages are excluded from the non-endocrine compartments, even at ovulation when the highly inflammatory microenvironment may favor their recruitment. As already mentioned, the OSE shows peculiar properties as compared with other FRT epithelia, with which it shares a common embryonal origin; one of such

peculiarities is the absence of interactions with macrophages, which are instead tightly intermingled with epithelial cells lining the endometrial surface and glands and the tubal wall (Gaytán *et al.*, 2007; King *et al.*, 2011). On the other hand, macrophages are found in association with ovarian epithelial cells when these are transformed into metaplastic cells and it is thus supposed that macrophages participate in ovarian carcinogenesis. Thus, it will be important to understand the role of macrophages in the ovarian endocrine activity and study the mechanisms that allow or inhibit these cells to communicate with FRT epithelia (Gaytán *et al.*, 2007).

Ovaries-specific phenotypes and functions

Along with the increase in cell number, fluctuations in estrogen levels associate with the acquisition of specialized functions by ovarian macrophages that are necessary for the maturation of oocytes and for the development, fate and vascularization of ovarian follicles.

Immune polarization and extracellular communication. Macrophages endowed with pro-healing and regenerative activities accumulate during the pre-ovulatory phase of follicle development and favor granulosa cell proliferation through the production of growth factors, such as bFGF, EGF and VEGF (Care *et al.*, 2013). On the other hand, the peri-ovulatory phase is associated with the increase of M1-like macrophages in the ovulating follicle. In fact, ovulation has been described as an inflammatory event that mainly enrolls inflammatory macrophages, which sustain the infiltration of additional immune cells, tissue disruption and subsequent maturation and functional specialization of granulosa cells through the secretion of inflammatory mediators (i.e. chemokines, reactive nitrogen species, prostaglandin $F_{2\alpha}$) and matrix remodeling enzymes (Espey, 1980; Machelon *et al.*, 1995; Nakao *et al.*, 2015; Shkolnik *et al.*, 2011; Wong *et al.*, 2002). Macrophage-derived signals are also important for vessel integrity of the antral follicle and *corpus luteum*, since whole body ablation of macrophages results in hemorrhage limitedly to the ovaries and not other tissues (Care *et al.*, 2013; Turner *et al.*, 2011). Apoptosis of granulosa and luteal cells is triggered by inflammatory mediators, including TNFα, while an increased macrophage number in the atretic follicle and *corpus albicans* has been associated with tissue regression and removal through the release of catabolic mediators and phagocytosis (Carlock *et al.*, 2014; Pate and Landis Keyes, 2001;

Shirasuna *et al.*, 2013; Stocco *et al.*, 2007; Wu *et al.*, 2015).

Thus, ovarian follicles are populated by functionally distinct subtypes of macrophages, as confirmed by the recent identification of ovarian macrophage subsets that differentially express antigen presentation and adhesion molecules (Carlock *et al.*, 2013). Importantly, a deranged balance between inflammatory and anti-inflammatory phenotypes has been proposed as a pathological link towards infertility and ovarian dysfunction (Uri-Belapolsky *et al.*, 2014).

Iron homeostasis. Non-heme iron in mouse ovaries is predominantly confined to macrophages, especially those adjacent to degenerating *corpora lutea* and apoptotic atretic follicles where ferrous ions are released (Asano, 2012). Both macrophages and the iron overload , derived from retrograde menstruation, are involved in the ceasing of ovarian function in women approaching the menopause, while dysfunctional iron handling by ovarian macrophages appears to contribute to malignant degeneration of the ovary (Vercellini *et al.*, 2011).

Cholesterol metabolism and steroidogenesis. The growing follicle is a site of cholesterol enrichment for its usage in steroidogenesis and incorporation in newly formed ovarian and granulosa cells. Indeed, the metabolism of cholesterol used for gonadal steroidogenesis drastically changes during the peri-ovulatory phase in association with changes in macrophage number and phenotype (see Figure 2). As shown in Figure 2, steroidogenesis in theca, granulosa and luteinizing cells is associated with resident macrophages showing an alternative polarization phenotype, while the sharp pre-ovulatory reduction in estrogen synthesis is linked to increased number of M1-like macrophages, which are known to inhibit steroidogenesis through the secretion of inflammatory cytokines, both in the ovaries and testes (Bornstein *et al.*, 2004; CHEN *et al.*, 1992; Leisegang and Henkel, 2018; Samir *et al.*, 2017). Although macrophages are well-established regulators of cholesterol homeostasis, the role and identity of mediators secreted by M2 macrophages as well as the ability to directly supply cholesterol for steroidogenic cells are still unknown. As already mentioned in the previous Section, estrogens are able to both stimulate cholesterol efflux in macrophages and induce their M2 polarization, suggesting that these cells might sustain estrogens synthesis in response to estrogens themselves. Interestingly, an increased number of lipid-laden macrophages are observed

particularly at sites of excess cholesterol accumulation and follicular atresia in the ovaries of female patients with congenital lipoid adrenal hyperplasia (lipoid CAH), an endocrine disorder linked to a defect in steroidogenesis and premature ovarian failure, suggesting a role for macrophages in cholesterol accumulation in the ovary (Ishii *et al.*, 2016). Nevertheless, cholesterol storage and usage by ovarian macrophages are still poorly defined to understand the impact of these cells on the physiopathology and estrogen dependence of ovarian endocrine activity.

Circadian rhythm. Clock genes expression in the ovary occurs in pre-antral follicles and further increases in the late antral and preovulatory stages in granulosa, theca and stromal cells and in oocytes (Fahrenkrug *et al.*, 2006; Karman and Tischkau, 2006). The circadian clock of the ovaries drives the expression timing of crucial proteins for ovarian physiology, such as LH receptor and steroidogenesis enzymes, demonstrating that the ovary plays an intrinsic role in the timing of female reproduction (Yoshikawa *et al.*, 2009; Nakamura *et al.*, 2010; Mereness *et al.*, 2016). Indeed, disruption of the ovarian circadian clock is associated with infertility and reproductive pathologies (Khan *et al.*, 2012; Simonneaux and Bahougne, 2015). It is increasingly evident that all events occurring during the reproductive cycle in females are rhythmically regulated by an integrated network of hormonal and circadian signals that derive from and operate in brain and FRT cells. Emerging evidence suggests that these signals regulate each other, as in the case of estrogen and clock gene expression in FRT, providing an additional level of control in reproductive synchrony; dangerous consequences for women's fertility and health may also emerge when impairment of this complex network occurs at any of its control levels (Simonneaux and Bahougne, 2015).

Macrophages in the oviducts

Cell distribution

Macrophages are localized within the epithelial, *lamina propria* and wall layer compartments of the human Fallopian tubes (Haney *et al.*, 1983; Ardighieri *et al.*, 2014).Macrophages have also been identified within the tubal lumen in close proximity with the cumulus cells complex that surrounds the oocyte (Akkoyunlu *et*

al., 2003; King *et al.*, 2011). Following ovulation, the fallopian tubes are acutely exposed to the follicular fluid that is enriched with inflammatory mediators (e.g. cytokines, ROS generating enzymes, proteases), which increase the number of macrophages in the tubal walls and their interactions with epithelial cells (King *et al.*, 2011). Contrary to epithelial cells of the endometrium, epithelial cells lining the oviduct walls do not proliferate in response to ovulation nor estrogens, but their DNA is frequently damaged by inflammation; importantly, epithelial cells in the distal part of the fallopian tubes may be sloughed by the inflammatory burden driven by ovulation and penetrate the ovarian surface together with macrophages, a mechanism that may be involved in ovarian cancer pathogenesis (Kurman and Shih, 2010; King *et al.*, 2011). Thus, inflammation and macrophages in the ovarian tubes have important functions for tissue homeostasis, although still poorly deciphered. Interestingly, female patients with inflammatory peritoneal disorders show higher levels of oviductal macrophages, suggesting that tubal homeostasis is also influenced by peritoneal inflammation (Haney *et al.*, 1983).

Oviduct-specific phenotypes and functions

Immune polarization and extracellular communication. The mucosal secretions and resident immune cells of the uterine tubes represent, like in other mucosal surfaces, protective mechanisms against microorganism invasion as well as key regulators of tissues homeostasis. Some evidence has shown increased inflammation and macrophage density in the tubal mucosa of women with ectopic implantation, infertility, infection spread and neoplastic transformation suggesting a role for macrophages in tubal cells motility and receptivity (Shaw and Horne, 2012; George *et al.*, 2016; Shao *et al.*, 2012; Tonello and Poli, 2007). Moreover, prolonged exposure to follicular and peritoneal fluid has been proposed as a causative mechanism promoting tubal tumorigenesis (Vercellini *et al.*, 2011; George *et al.*, 2016). However, little information is available on the role of macrophages in tubal epithelial cells secretory function and the healthy and safe migration and fertilization of the oocyte within uterine tubes.

Macrophages in the uterus

Cell distribution

Macrophages are non-uniformly scattered throughout the endometrium and their density changes under the influence of hormonal fluctuations. Figure 2 summarizes the data obtained in women and rodent models, which showed that macrophages are mainly confined to the superficial endometrial stroma during the repair and proliferative phases, with a preferential distribution around or even within superficial endometrial glands, with no tendency to aggregate around vessels; their density then significantly rises in the late secretory phase in women or at *diestrus* in mice (Stewart and Mitchell, 1991; Shimada-Hiratsuka *et al.*, 2000; Russell *et al.*, 2011, 2013; Thiruchelvam *et al.*, 2013; Cousins *et al.*, 2016). Specific sets of chemokines are released by the epithelial, stromal, immune and vascular compartments with differences at each of these sites according with the ovarian phase (Sanford *et al.*, 1992;(MacDonald *et al.*, 2010; Thiruchelvam *et al.*, 2013). Macrophages are also found in the myometrium, where their number remains constant throughout the ovarian cycle. During the proliferative phase macrophages seem to derive from the amplification of resident cells; interestingly, macrophage precursor cells are also present in the mouse uterus and depend on ovarian steroid hormones for replication (Hudson Keenihan and Robertson, 2004). On the other hand a transient influx of monocytes and monocyte-derived macrophages sustains the increase in cell density in the late secretory phase (Cousins *et al.*, 2016). The presence of macrophages in the shed endometrium and denuded luminal surface not only suggests their direct involvement in tissue destruction and repair but also indicates that at least some of these cells are not shed away during tissue remodeling. This opens the important question, still barely addressed, related to the mechanisms that remove macrophages to reduce their number. Macrophages may leave the endometrium by trafficking to the lymph nodes, although endometrial lymphatic circulation is poorly developed possibly to protect the female's immune system against autoantigens (Red-Horse, 2008), or by moving to endometrial lymphoid aggregates. These recently described structures have unknown functions but contain macrophages in a greater number at the secretory phase (Red-Horse, 2008; TABIBZADEH, 1990; Wira *et al.*, 2014). In addition, monocytes may be cleared by apoptosis following completion of endometrial repair, as recently suggested (Cousins *et al.*, 2016).

Thus, as in the ovaries and ovarian tubes, macrophages in the endometrium show preferential locations

and specific cellular connections, and are locally renewed from circulating precursors in response to ovarian inputs at each new cycle.

Macrophages within the uterine lumen. The tissue of origin of macrophages and other immune cells found in the uterine and cervical fluids has not been defined yet. Inflammatory cytokines are secreted into the uterine lumen by the apical compartments of luminal epithelial cells. It is not known yet whether these molecules attract macrophages from the lumen to the epithelial wall, where they could integrate in the macrophage endometrial compartment.

Uterus-specific phenotypes and functions

Histological and cytometric analyses in human and murine uteri allowed appreciating the existence of distinct phenotypic subsets of macrophages preferentially located in close proximity to exocrine glands and to areas of tissue remodeling, therefore believed to participate in mucosal function as well as in tissue degradation, repair and regeneration (Thiruchelvam *et al.*, 2013). As it occurs during the wounding and healing of other mucosae, shedding and reconstruction of the endometrial tissue require a series of well-controlled events that accelerate re-epithelialization and inflammation without scar or fibrosis formation; macrophages participate in all stages of wound healing and tissue repair (Smigiel and Parks, 2018). As discussed below, novel experimental models now allow to mimic human menstruation in mice (Cousins *et al.*, 2014); however, animal models with whole-body depletion of macrophages are not suited for studying the endometrium due to its functional dependence upon the hypothalamus-pituitary-ovarian axis that is interrupted by macrophage depletion (see Table 1). To circumvent this problem, ovariectomy is generally performed in female mice and, after few days of estrogen conditioning, a single E_2 administration is used to assess a proliferative response of endometrial cells. These experimental conditions have been used e.g. by Care et al. in *CD11b-DTR* females to assess the contribution of macrophages to hormone action (Care *et al.*, 2014). Although the results showed a dispensable role for macrophages in the estrogen-induced proliferation of differentiated epithelial cells of the endometrium, this experimental setting appears limited in evaluating the contribution of endometrial progenitor cells, although it is known that their regenerative potential sustains endometrial reconstitution through repeated proliferation and differentiation cycles

(Gargett *et al.*, 2015; Janzen *et al.*, 2013). Endometrial precursor cells expand under the positive regulation of estrogens and progesterone; as expected, the number of epithelial and leucocyte progenitor cells is reduced in the endometrium of ovariectomized mice (Deane *et al.*, 2016). Nevertheless, the responsiveness of resilient stem cells to estrogen signaling is still uncertain; further studies and models are needed to better understand estrogen action and their cellular targets in the endometrium.

Immune polarization and extracellular communication. During the proliferative phase, endometrial macrophages express membrane proteins (i.e. TFRC, CD69 and IntraCellular Adhesion Molecule-1, ICAM1), matrix remodeling molecules and growth factors that induce a permissive environment and allow the regeneration of tissue and ECM in preparation for fertility (Eidukaite and Tamosiunas, 2004; Salamonsen and Woolley, 1999; Thiruchelvam *et al.*, 2013). On the other hand, during the secretory phase macrophages generate a local inflammatory response *via* the release of cytokines (e.g. MIP1β/CCL4 and MIF) that either permits embryo implantation during the so-called "window of implantation" or induces uterine shedding, an event that further culminates in menstruation only in some primates, including women (Thiruchelvam *et al.*, 2013). *In vivo* studies using artificially induced menstruation in mice recently allowed to demonstrate that inflammatory monocytes and monocyte-derived macrophages are recruited during the simultaneous phases of tissue breakdown and repair to perform phagocytosis of apoptotic endothelial cells and tissue debris along with resident macrophages (Cominelli *et al.*, 2014; Cousins *et al.*, 2016). Transcription factors linked to phenotypic activation in macrophages, such as members of the KLF family, are highly expressed in reproductive tissues and have also been involved in endometrial and FRT pathologies (Daftary *et al.*, 2013; Simmen *et al.*, 2015).

Hemostasis and beyond. The relevance of hemostasis in the human endometrium is well established. The cessation of menstrual bleeding and subsequent reconstruction of functional endometrium are accompanied by the expression of coagulation factors, induction of platelet aggregation and fibrin deposition, under the influence of the local inflammatory and hormonal environment, while the reduction in tissue factor and thrombin levels creates a pro-hemorrhagic and fibrinolytic milieu that is associated with endometrial sloughing (Davies and Kadir, 2012). Importantly, altered expression of hemostatic factors

appears to be involved in endometriosis (Schatz *et al.*, 2016). Mostly investigated during pregnancy and labor, the contribution of macrophages to hemostasis in reproductive cycles is still ill defined.

Extracellular communication. Breakdown of the functional endometrial layer recruits macrophages mainly through the activity of MMPs and plasminogen activator, whose expression is upregulated during the menstrual phase in macrophages and other uterine cells (Jeziorska *et al.*, 1996; Thiruchelvam *et al.*, 2013). Whether the hormone-induced activation of VEGF-A mediated by ERα in macrophages is involved in the activity of these cells on vascular permeability and remodeling still needs to be clarified (McLaren *et al.*, 1996; Kanda and Watanabe, 2002; Pepe *et al.*, 2017). Through the secretion of factors, such as IL-6, affecting the glycosylation pattern of membrane proteins, uterine macrophages also regulate the ability of uterine epithelial cells to create a receptive surface for embryo implantation (Nakamura *et al.*, 2012).

Iron homeostasis. Many genes related with iron homeostasis are up-regulated in the mouse uterus during endometrial growth and proliferation induced by pharmacological treatment with estrogens, suggesting an important role for estrogens in iron metabolism, possibly to meet the increased iron demand by replicating endometrial cells during the proliferative phase (Stuckey *et al.*, 2006). These cells may also include ovarian macrophages that grant iron availability for surrounding endometrial cells and for their own renewal and phenotypic adaptation. Iron handling by macrophages is also important for mucosal immunity, since iron proteins are also secreted into the uterine luminal fluid, and to buffer iron overload associated with retrograde menstruation and endometriosis in women (Defrere *et al.*, 2008).

Macrophages in the lower genital tract

The cervicovaginal mucosa is a specialized immune organ that preserves fertility by promoting tolerance to paternal antigens and by protecting against genital pathogens (Zhou *et al.*, 2018). Less information is available on the physiology and endocrine regulation of macrophages that populate the lower genital tract (LGT), namely the cervix and vagina, in non-pregnant, healthy females.

Cell distribution

Macrophages are a dominant population among vaginal and cervical innate immune cells, with some differences among these anatomical regions (Pudney *et al.*, 2005). In contrast to the upper FRT, their number appears almost stable throughout the menstrual cycle with a slight increase in the cervical mucosa during the menstrual phase, even though high intra and inter-subject variability has been reported (Pudney *et al.*, 2005; Trifonova *et al.*, 2014). Histological observations of the mouse vaginal fold showed that the vaginal mucosa undergoes extensive modifications in the number of leukocytes, which are absent at proestrus and estrus while present at metestrus and diestrus (Gal *et al.*, 2014). Interestingly, inflammatory mediators that are present in seminal fluid, such as cytokines and prostaglandins, increase substantially the number of macrophages and other immune cells in the epithelium and stroma of human cervix and uterus after coitus, further suggesting a role for inflammatory cells in promoting fertility (Adefuye *et al.*, 2016; Sharkey *et al.*, 2012).

LGT-specific phenotype and functions

Since cervical macrophages contribute to the remodeling of the LGT during parturition and represent a major cellular target for viral infections in women, these cells have been intensely studied for their immune functions in pregnancy-associated diseases or sexually-transmitted infections. This research allowed appreciating the functional specialization of vaginal macrophages, as indicated by the higher expression levels of CXCR4, the HIV-1 receptor, as compared to those residing in other mucosae such as intestinal macrophages (Barreto-de-Souza *et al.*, 2014; Roan and Jakobsen, 2016; Shen *et al.*, 2009). Interestingly, vaginal and cervical macrophages preferentially reside along the stroma-epithelium interface; it has been suggested that these cells migrate towards the epithelium or even into cervicovaginal secretions (Pudney *et al.*, 2005), to capture and disseminate HIV infection through CXCR4 activity (Olesen *et al.*, 2016). However, little is known on the ontogeny and specific functions of LGT macrophages beyond their role in immunity against infections (Iijima *et al.*, 2008).

Immune polarization and extracellular communication. The composition of inflammatory and defense-related proteins (defensins) in the vaginal and cervical mucus varies during the menstrual cycle, with their increased expression being strongly correlated with decreased HIV infectivity and their dysregulation

associated with reproductive pathologies in women (Grande *et al.*, 2015, 2017; Hughes *et al.*, 2016). In the cervical tissue of healthy mice, estrogen has been shown to modulate the expression of inflammatory genes, such as IL-1β and the S100 calcium binding protein A9 (S100a9) in vaginal macrophages and dendritic cells by ERα-dependent pathways. Subsequent activation of epithelial cells and differentiation of Th17 cells lead to enhanced anti-viral responses in the genital tract (Polan *et al.*, 1988; Stygar *et al.*, 2007; Anipindi *et al.*, 2016).

Thus, although only marginally addressed, estrogens action in LGT macrophages is clearly associated with functional responses.

Macrophages and FRT pathologies

Gynecological dysfunctions and cancer

Emerging evidence indicates that ovarian dysfunction and diseases are associated with impaired activity of ovarian macrophages. During senescence, fibrotic transformation of ovarian tissue is accompanied by accumulation of multinucleated macrophages with enhanced phagocytic function and production of pro-inflammatory factors (Asano, 2012; Briley *et al.*, 2016). Activated macrophages with poorly characterized phenotypes are also found in the follicular fluid of patients suffering from premature ovarian failure and polycystic ovary syndrome (Bukovsky and Caudle, 2008, 2012). Macrophages with M2-skewed phenotype known as tumor-associated macrophages (TAMs) are detected in several tumors including gynecological cancers. TAMs show immunosuppressive and pro-tumorigenic effects and are intensely studied to understand disease progression and to identify novel anticancer agents (Krishnan *et al.*, 2018). However, potential stimulatory effects on tumor growth specifically dictated by estrogen-induced TAMs have not been elucidated.

3.5.2 Endometriosis

Endometriosis is a gynecological disorder characterized by ectopic growth of endometrial tissue fragments on the surface of the peritoneum and ovaries, causing pelvic pain and infertility. Endometrial cells have access to the peritoneal cavity via retrograde migration through the Fallopian tubes and adhesion and

invasion of the mesothelial cell layer of the peritoneum (Young *et al.*, 2013). Ectopic endometrial lesions are enriched with macrophages derives from both the shed tissue itself and the peritoneal and vascular compartments. Under the influence of endometriosis-associated pathologic signals, including hypoxia, iron overload and inflammation, macrophages become reprogrammed to operate in favor of lesion development, as suggested by a derangement in immune polarization, phagocytosis and vascular activity of macrophages and by their preferential location, in analogy with the endometrium, as single or aggregated cells in close proximity to glandular structures in endometriotic tissue (Greaves *et al.*, 2014; McLaren *et al.*, 1996, 1997; Nakamura *et al.*, 2012). A heterogeneous population of potentially dangerous pro-inflammatory and anti-inflammatory macrophages is present within or around the lesions, since pro-angiogenetic, matrix remodeling, iron-recycling and growth factors produced by M2 macrophages sustain endometriotic lesion development and interactions with vasculature and nerve fibers, while M1 macrophages enable early initiation of endometriosis and sustains stromal cell activity *via* released pro-inflammatory molecules, such as IL-6, TNF-α or prostaglandin E_2 (Lin *et al.*, 2006; Bacci *et al.*, 2009; Tran *et al.*, 2009; Capobianco *et al.*, 2011; Capobianco and Rovere-Querini, 2013; Khan *et al.*, 2015; Yuan *et al.*, 2017; Burns *et al.*, 2018).

The ectopic endometrial tissue retains the ability to respond to sex steroid hormones and undergoes destruction and remodeling during the menstrual cycle, although this endocrine signaling is somehow modified in endometriosis, as suggested by elevated estrogen levels, progesterone resistance and altered expression of ERs, PR and coregulators, and possibly by the limited therapeutic efficacy of hormonal drugs (Han *et al.*, 2015; Han and O'Malley, 2014; Nasu *et al.*, 2011; Szwarc *et al.*, 2014; Zhao *et al.*, 2015). The use of novel mouse models of menstruation and endometriosis will allow a better understanding of estrogen-macrophage interplay in endometriosis, as already suggested for innervation events of early lesions development in animal models of disease (Greaves *et al.*, 2015; Burns *et al.*, 2018). Thus, current data suggest that the estrogen-macrophage interplay has a relevant impact on endometriosis through the amplification of macrophages bearing a permissive phenotype for endometrial cell proliferation, vascularization and innervation. Current therapeutic interventions in endometriosis make use of

progesterone, an off-signal of estrogen activation, to oppose estrogens actions in endometrial cells; being insensitive to progesterone, macrophage responses to estrogens are probably unaffected by such therapies, hinting at appropriate antagonists of macrophage estrogen signaling as novel therapeutic agents in endometriosis.

Discussion

The distribution at specific locations in reproductive tissues, interaction with selected cell types and acquisition of distinct phenotypes and specialized functions strongly substantiate the hypothesis that macrophages are key players in the homeostasis and rhythmical renewal of the FRT. Importantly, the specificity of the intercellular communications between macrophages and FRT cells, although still poorly addressed, may induce phenotypically distinct subsets of macrophages that express specific mediators, thus representing candidate therapeutic targets for infertility or FRT diseases. The peculiar ability of macrophages to adapt and respond to diverse signals allows them to actively participate in the coordination of reproductive events by translating endocrine signals, such as estrogens or glucocorticoids, and local cues, such as cytokines or hypoxia, into specific cellular interconnections that are precisely organized in time and space, as summarized in Figure 3A. The endocrine communication between macrophages and reproductive tissues is mainly driven by estrogens, whose function is associated with diverse responses of FRT macrophages. The physiological meaning of this interplay might be to generate a tolerant environment for egg movement, fertilization and implantation as well as to sustain a highly reactive and renewable system for the cyclic remodeling of reproductive tissues. Accordingly, derangements of macrophage function and responsiveness may be involved in estrogen and macrophage-dependent gynecological diseases, such as uterine cancer and endometriosis (Figure 3B). A better understanding of the molecular and cellular mechanisms that allow macrophages to participate in the homeostasis of reproductive cycles and to act as estrogen-responsive cells will provide new knowledge and potential pharmacological targets for reproductive procedures and for estrogens and macrophage-dependent gynecological diseases.

AUTHORS' ROLES

G.P., F.M. and E.V. performed literature search; G.P., F.M. and E.V. conceived and drafted the manuscript; E.V. and S.D.T. prepared the figures; G.P., L.M., S.D.T., A.M., A.C., and E.V. contributed to the interpretation and critical discussion of the data; all authors revised the manuscript and approved the final version.

FUNDING

European Union's Seventh Framework Program (FP7/2007-2013; n. 278850 INMIND); CARIPLO Foundation (n. 2011-0591).

CONFLICT OF INTEREST

The authors declare no competing financial interests

FIGURE LEGENDS

Figure 1. Molecular mechanisms of estrogen action and macrophage responses.

Estrogens are the only female sexual hormones that directly communicate with macrophages, since these cells express ERα and GPER1 but do not express progesterone, LH or FSH receptors. Estrogens-activated ERα dimerizes and translocates to the nucleus where it regulates target gene transcription by binding to short DNA sequences known as estrogen responsive elements (EREs), within gene promoters and by recruiting chromatin protein complexes and transcriptional coregulators (CoR). Genomic responses may also derive from ERα interference with the expression or activity of other transcription factors, such as NF-κB and C/EBP, as well as by a reduced availability of transcriptional co-regulators. Hormone-activated ERα and GPER1 also directly induce cytoplasmic responses, including PI3K and MAPK activation, calcium mobilization, and cAMP formation. Under physiological conditions, estrogen action in macrophages mediates several biological processes, which are overall associated with the induction of a tolerant immune environment for the growth, specialization and remodeling of surrounding cells and tissues.

Figure 2. Distribution, phenotype and functions of FRT macrophages.

Female reproductive tissues are colonized by distinct populations of M1 and M2 macrophages. In the upper FRT, these cells change in number, distribution and function in association with estrous cycle phases and fluctuations in estrogens levels. Macrophages with M2-like activities are more abundant during the pre-ovulatory phase and also found in the *corpus luteum*; inflammatory macrophages sharply increase immediately before ovulation in the ovaries and at the end of the ovarian cycle in the endometrium and generally predominate in tissues during the post-ovulatory phase. In the lower FRT, macrophages remain more constant and have mainly been associated with defensive mechanisms against pathogens invasion. Beyond this immune task, macrophages in the upper FRT participate in specific processes (shown in italics), such as proliferation, differentiation and apoptosis of granulosa cells (GC), endocrine activity, ovulation and vascularization in the ovaries, epithelial cells (EC) proliferation and secretory activity in the oviducts and endometrium, where they also regulate extracellular matrix (ECM) and vascular remodeling.

Figure 3. Macrophage cellular interconnections in the homeostasis of the FRT.

A, Macrophages establish physical contacts and functional connections with FRT cells, such as epithelial, endocrine and immune cells, which are precisely organized in space and time under the influence of endogenous hormones, such as estrogens or glucocorticoids, and local signals, including cytokines or hypoxia. The responsiveness of macrophages to estrogens occurs both directly, through ERs expressed in macrophages, and indirectly, *via* estrogen-regulated cytokines-mediated pathways. **B,** The responsiveness of macrophages to estrogens contributes to FRT functions, while any alterations in macrophage functions or estrogens signaling might promote and sustain estrogens and macrophage-dependent reproductive pathologies, such as infertility, ovarian cancer and endometriosis.

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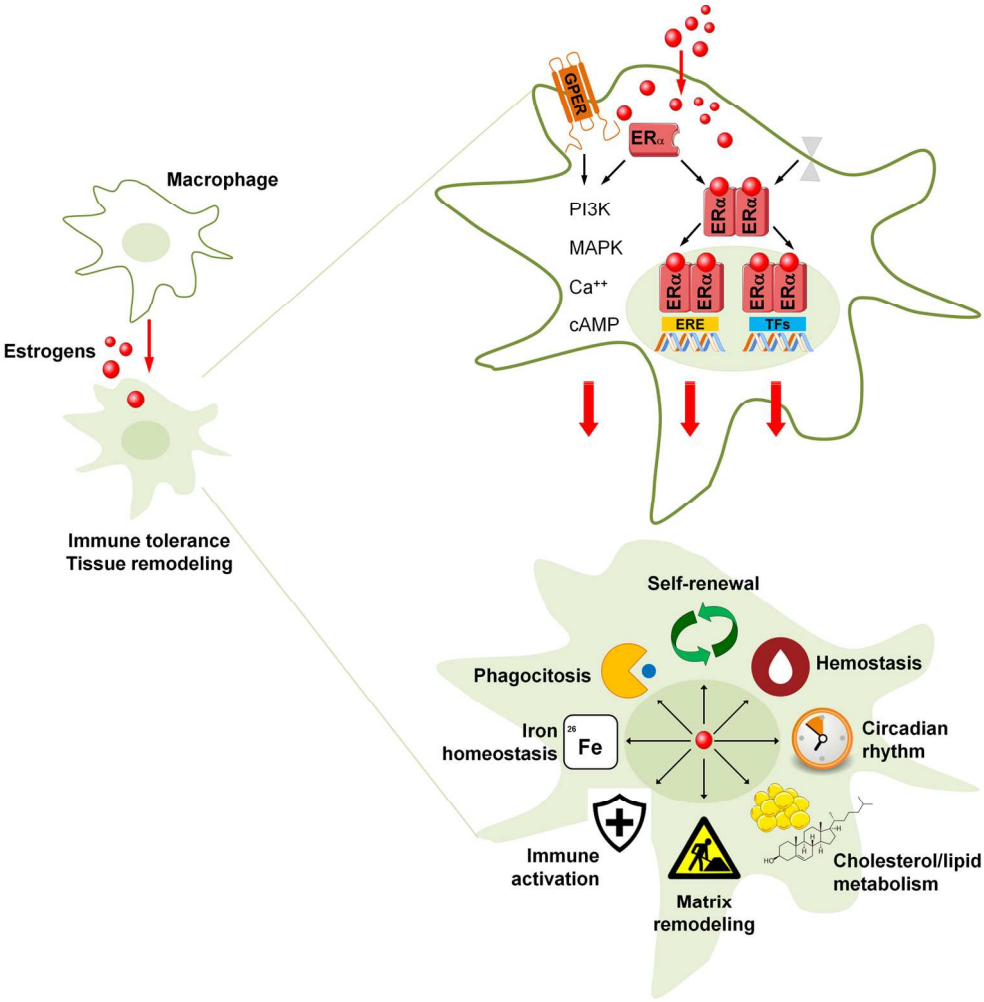

Figure 1. Molecular mechanisms of estrogen action and macrophage responses.
Estrogens are the only female sexual hormones that directly communicate with macrophages, since these cells express ERα and GPER1 but do not express progesterone, LH or FSH receptors. Estrogens-activated ERα dimerizes and translocates to the nucleus where it regulates target gene transcription by binding to short DNA sequences known as estrogen responsive elements (EREs), within gene promoters and by recruiting chromatin protein complexes and transcriptional coregulators (CoR). Genomic responses may also derive from ERα interference with the expression or activity of other transcription factors, such as NF-κB and C/EBP, as well as by a reduced availability of transcriptional co-regulators. Hormone-activated ERα and GPER1 also directly induce cytoplasmic responses, including PI3K and MAPK activation, calcium mobilization, and cAMP formation. Under physiological conditions, estrogen action in macrophages mediates several biological processes, which are overall associated with the induction of a tolerant immune environment for the growth, specialization and remodeling of surrounding cells and tissues.

160x158mm (300 x 300 DPI)

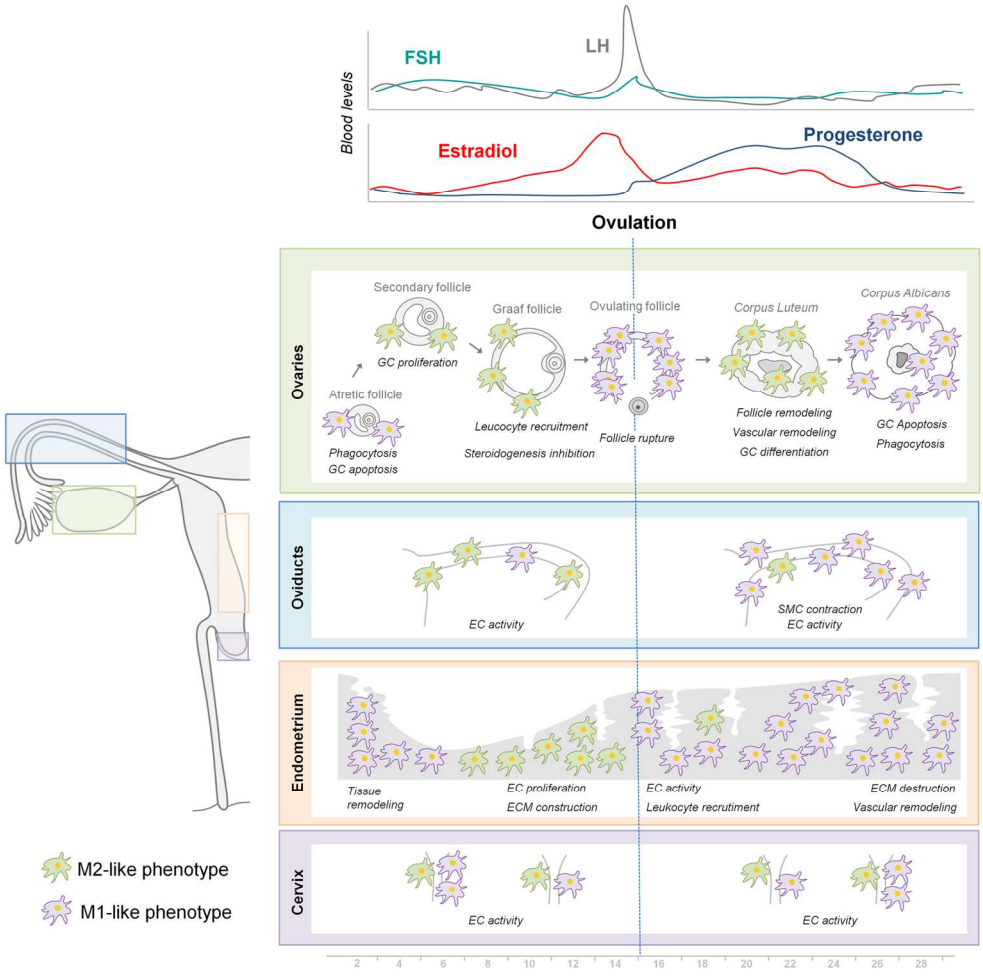

Figure 2. Distribution, phenotype and functions of FRT macrophages.
Female reproductive tissues are colonized by distinct populations of M1 and M2 macrophages. In the upper FRT, these cells change in number, distribution and function in association with estrous cycle phases and fluctuations in estrogens levels. Macrophages with M2-like activities are more abundant during the pre-ovulatory phase and also found in the corpus luteum; inflammatory macrophages sharply increase immediately before ovulation in the ovaries and at the end of the ovarian cycle in the endometrium and generally predominate in tissues during the post-ovulatory phase. In the lower FRT, macrophages remain more constant and have mainly been associated with defensive mechanisms against pathogens invasion. Beyond this immune task, macrophages in the upper FRT participate in specific processes (shown in italics), such as proliferation, differentiation and apoptosis of granulosa cells (GC), endocrine activity, ovulation and vascularization in the ovaries, epithelial cells (EC) proliferation and secretory activity in the oviducts and endometrium, where they also regulate extracellular matrix (ECM) and vascular remodeling.

146x146mm (300 x 300 DPI)

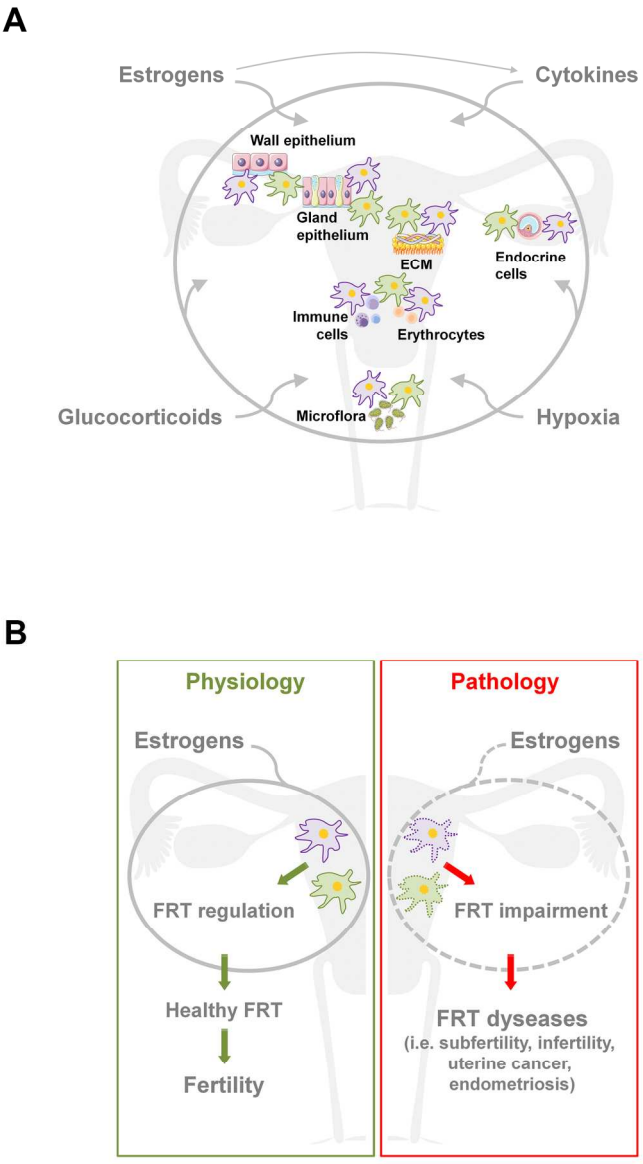

Figure 3. Macrophage cellular interconnections in the homeostasis of the FRT.
A, Macrophages establish physical contacts and functional connections with FRT cells, such as epithelial, endocrine and immune cells, which are precisely organized in space and time under the influence of endogenous hormones, such as estrogens or glucocorticoids, and local signals, including cytokines or hypoxia. The responsiveness of macrophages to estrogens occurs both directly, through ERs expressed in macrophages, and indirectly, via estrogen-regulated cytokines-mediated pathways. B, The responsiveness of macrophages to estrogens contributes to FRT functions, while any alterations in macrophage functions or estrogens signaling might promote and sustain estrogens and macrophage-dependent reproductive pathologies, such as infertility, ovarian cancer and endometriosis.

150x230mm (300 x 300 DPI)

Table 1. Steroid receptors expression in macrophages

Macrophage source	mRNA content						
	ERα (ESR1)	**ERβ (ESR2)**	**GPER (GPER1)**	**PR (PGR)**	**AR**	**GR (NR3C1)**	**RPLP0**
Peritoneal macrophages*	1.4	nd	0.08	nd	nd	30	1290
Peritoneal macrophages**	151	nd	nd	nd	34	2821	52333
Monocyte-derived macrophages***	110	nd	20	nd	45	1180	12000

Expression levels of steroid receptor transcripts detected in different macrophage datasets. Gene names are reported in brackets.

* BioProject ID PRJNA376257, reported in Pepe et al., 2016. Data refer to murine peritoneal macrophages from adult female mice and are expressed as reads per kilobase of transcript per million mapped reads.

** GEO dataset ID GSE107174. Data refer to murine peritoneal macrophages and are expressed as reads per kilobase of transcript per million mapped reads. Mouse sex is not specified.

*** GEO dataset ID GSE5099, reported in Martinez et al., 2006. Data refer to in vitro differentiated monocyte-derived macrophages from men and women healthy donors and are expressed as arbitrary units at net of background level (20).

Abbreviations: MDM, monocytes-derived macrophages; nd, not detected; AR, androgen receptor; GR, glucocorticoid receptor; RPLP0, ribosomal protein lateral stalk subunit P0 (house-keeping gene).

Table 2. Reproductive phenotypes in macrophage-depleted mouse models

Mouse models		Reproductive and endocrine phenotypes in adult females	FRT phenotype			
			Ovaries	**Endometrium**	**Notes**	**References**
conditional	**Clodronate liposomes**	Not described	Reduced ovulation rate. Extended duration of M/DE stage	No MP depletion	Intrabursal injections reduce theca MP. No liposomal diffusion through the endometrium	Van der Hoek et al., 2000
	Mab against CSF1R	Estrous cycle is present. Cycle onset and phases duration not described.	No MP depletion (complete MP ablation in testis)	No MP depletion	No reduction of blood monocytes	MacDonald et al., 2010; Sauter et al., 2014
	CD11b-Dtr	Infertility when MP are depleted after ovulation, as a result of failure to form *corpora lutea* and to synthesize progesterone. Embryo implantation inhibited by MP depletion after conception, rescued by progesterone administration.	Hemorrhages. Loss of integrity of vessels and basal membranes in antral follicles and corpus luteum.	E_2-induced epithelial cell proliferation in ovx mice unaffected. Endothelial cell number in ovx mice unaffected.	Significant MP reduction in ovaries and uterus	Turner et al., 2011; Care et al., 2013; Care et al., 2014
constitutive	***Csf1^{op}/Csf1^{op}***	Reduced fertility. Delayed microglial colonization of the hypothalamus during development; alteration of neuronal circuitries governing feedback sensitivity of GnRH neurons. Reduced ovulatory frequency and number. Low pregnancy rates. Absence of mammary gland branching after parturition; females unable to nurture their pups. Absence of E_2 surge at P, normal E_2 levels at E, M and DE. Generally severe growth and endocrine defects	Defective follicular development. Defective ovulation. Delayed cycle onset. Prolonged cycle length (mainly stopped in ME).		Significant MP reduction in antral follicles	Cohen et al., 1992; Cohen et al., 2002
	Csf1r$^{-/-}$	Reduced fertility	Prolonged cycle length (mainly stopped in ME)		Blood monocyte reduction	Dai et al., 2002

MP, macrophages; Mab-α, monoclonal antibody; E_2, 17β-estradiol; ovx, ovariectomized; P, proestrus; E, estrous; M, metestrous; DE, diestrous